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Molecular-Intelligence Correlations in Young Fragile X Males With a Mild CGG Repeat Expansion in the FMR1 Gene

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Several mechanisms can explain the occurrence of full-mutation fragile X males with an IQ level above −2 SD below mean, also called "high-functioning fragile X males." Incomplete methylation of the CpG island at the 5′ end of the FMR1 gene is one of these mechanisms. The present study describes the physical and behavior phenotypes in 7 fragile X boys with CGG repeat insertions in the FMR1 gene between 600–2,400 base pairs. The degree of methylation at the FMR1-associated CpG island ranges in peripheral blood lymphocytes from 0–95%. Subjects with a low degree of methylation at this site have mild or absent physical characteristics of the fragile X syndrome, while subjects with a high degree of methylation at this site have more severe physical characteristics. In this range of CGG repeat insertion (600–2,400 base pairs), the degree of methylation at the FMR1-associated CpG island is a good predictor of intelligence, while CGG repeat insertion length is not.

KEY WORDS: fragile X syndrome, high-functioning males, methylation, FMR1 gene

INTRODUCTION

In the first descriptions of fragile X syndrome, mental retardation was considered an absolute characteristic of fragile X hemizygotes. Later, transmitting males were found in whom mental retardation and other characteristics of fragile X phenotype were absent, i.e., the so-called normal transmitting males [Daker et al., 1981; Sherman et al., 1985]. After the molecular basis of the fragile X mutation was described [Verkerk et al., 1991], the normal transmitting males were found to have a shorter CGG repeat insertion at the FMR1 gene, and the differentiation was made between a premutation, with an insertion of <600 base pairs, and a full mutation, with a longer insertion. Most males with a full mutation in the FMR1 gene have fragile X syndrome. Besides physical characteristics and often characteristic behavior traits, the fragile X phenotype includes mild, moderate, or even severe mental retardation [Borghgraef et al., 1987, 1990].

Only recently, fragile X males with a full mutation have been described with an intelligence level in the so-called borderline or even normal range [Hagerman et al., 1994; Rousseau et al., 1994a; Steyaert et al., 1994]. These IQ ranges are defined as between −2 and −1 SD below mean for borderline range, and IQ higher than −1 SD below mean for normal range. Hagerman et al. [1994] found mild physical signs of fragile X syndrome in most of these males. Several mechanisms may explain this phenomenon. First, intelligence is a characteristic influenced by many genes. Thus, as in any population, in a population of fragile X males the distribution of intelligence level follows a bell-shaped curve. However, in this particular population, the mean is displaced to the left and is around an IQ of 40, instead of IQ 100, as in the total population. A sporadic individual with a complete FMR1 mutation could be at the far right end of the curve and have an IQ in the normal range. However, in studies on large groups of fragile X males, Rousseau et al. [1994b] found no males with a full mutation and normal intelligence, and Hagerman et al. [1994, subjects 17 and 24] reported only 2/29 "high-functioning" fragile X males with a full FMR1 mutation and complete methylation at the FMR1-associated CpG island.

Another mechanism to explain the occurrence of "high-functioning" fragile X males is somatic mosaicism of the CGG-insertion. In one tissue, e.g., peripheral lymphocytes, mutation and premutation can occur simultaneously, as well as different lengths of full mutation or premutation. This instability can give either two distinct types of cells in a particular tissue (full mutation-premutation mosaicism), or a whole spectrum of inser-

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tion lengths, showing a smear on Southern blot. Fragile X males with a mosaic pattern of CGG insertion length in peripheral lymphocytes seem to have a better average IQ than subjects with only full mutation [Hagerman et al., 1994].

A third proposed possibility is that mosaicism for the length of insertion may not only occur within one tissue, but also between different tissues. Consequently, while in peripheral lymphocytes or another tissue a full mutation is found in a subject, it is possible that the brain cells of this subject carry only the premutation and hence produce FMR1 protein and allow the subject to have a normal IQ. However, until now little has been learned about the importance of mutation differences between different tissues and hence, whether this mechanism contributes significantly to the occurrence of "high-functioning" fragile X males. For obvious reasons, it is not possible to evaluate the insertion length in brain cells of subjects.

A fourth mechanism has been described recently: in some "high-functioning" fragile X males [Rousseau et al., 1994a; Hagerman et al., 1994; Steyaert et al., 1994], methylation of the CpG island at the 5′ end of the FMR1 gene is found in only a fraction of the cells with a full mutation. The other cells with the full mutation show no methylation at this locus. As methylation represses FMR1 transcription [Sutcliffe et al., 1992], one could expect some FMR1 protein to be produced in subjects with a full mutation but partial methylation. Hagerman et al. [1994] demonstrated FMR1 protein production in two of their "high-functioning subjects." In these subjects, the amount of FMR1 protein produced was limited, probably because the FMR1 mRNA was available only in a limited number of cells. And even in these cells with full mutation and no methylation, a slowdown of FMR1 mRNA translation can play a role in limited protein production, as recently illustrated by Feng et al. [1995].

Combinations of these different mechanisms operating in one subject are also possible, making the molecular-phenotypical relations only more complex. The present study analyzes the effect of partial methylation of the FMR1-associated CpG island in a group of boys with a full CGG repeat expansion in the FMR1 gene.

MATERIALS AND METHODS

Subjects

We found 7 boys, between 7–15 years old, with expanded CGG insertions in the FMR1 gene and with an incomplete degree of methylation of the FMR1 gene-associated CpG island. The subjects come from five unrelated families: 5 of them are index patients who presented at the outpatient clinic (subjects 2, 4, 5, and 7) or were seen in an institution for the mentally retarded (subject 6). The 2 others (subjects 1 and 3) are sibs in two different families. One of the sibs (subject 3) was known to have learning difficulties, and attention and behavior problems, years before fragile X was detected in his younger brother (subject 7). In the other pair of sibs, the sib who was not an index subject (subject 1) had not been reported as having problems before detection of fragile X in his brother (subject 5).

Six of the 7 subjects underwent physical examination in our center. The seventh subject (subject 6) underwent physical examination elsewhere, and his physical phenotype will not be included in the data. Any degree of fragile X phenotype will be reported as "phenotype present." The subjects were also seen for intelligence testing with the revised Wechsler Intelligence Scale for Children (WISC-R), Dutch or French version.

Next we took a behavior and psychiatric history of all subjects. In 5 children the Achenbach's Child Behavior Checklist, Dutch version [Verhulst et al., 1990], was also taken. We scored as characteristic for fragile X behavior phenotype only the combination of attention deficits/hyperactivity and some form of shyness or social ambivalence. We also scored "behavior phenotype present" in two adolescent subjects (subjects 2 and 3) in whom these problems had occurred in earlier phases of development, but were not clinically significant anymore.

Molecular Data

FMR1 status was determined on DNA analysis of peripheral lymphocytes. Southern blot analysis using probe St12.3 was performed after double digestion with *Eco*RI and *Eag*I. When multiple bands of insertion length, or a smear, were found, statistical analysis was performed separately with the minimum and the maximum insertion length. Degree of methylation was determined by densitometry on the Southern blot radiograph. When only a thin streak of methylated DNA and a dense zone of unmethylated DNA were found, limits of precision of densitometry forced us to consider this arbitrarily as methylation in approximately 5% of the cells.

RESULTS

Phenotypic Characteristics (Table I)

All but 1 boy of the 6 who were clinically examined had physical characteristics of fragile X syndrome. In subjects 2 and 3 this involved only macroorchidism. Subject 4 had craniofacial characteristics and long ears. Subjects 5 and 7 had the full fragile X phenotype.

Full scale WISC-R (Table I) intelligence quotient (FSIQ) in subjects ranged from 53–119, with an average of 78. Subjects 5–7 go to special education schools. In the sib pairs we found large differences in intelligence level: in the pair subject 1/subject 5 the difference was 63 IQ points or 4 SD (in the WISC-R, 15 IQ points is 1 SD), and in the pair subject 3/subject 7, 30 IQ points or 2 SD.

Five of the 6 boys who underwent psychiatric examination have or have had behavior characteristics associated with fragile X syndrome. Subjects 2 and 3 were known as hyperactive preschoolers and primary school children, though this has disappeared at puberty in both. Subject 3 was treated for attention deficit hyperactivity disorder before fragile X was known in the family. The mutation was found in this family only later, through subject 3's younger brother, subject 7. Subjects 2 and 3 have had problems in socializing with peers, with a tendency to withdraw in both. Subject 3 still has

TABLE I. Summary of Molecular and Phenotypical Data in 7 Boys With Fragile X Mutation*

Subject number	Age	Full-scale WISC-R IQ	Methylation (%)	Insertion (kb)	Phenotypical features present	Behavioral features present
1	10	119	0	0.60	No	No
2	13	87	13	0.90 (smear)	Yes	Yes
3	16	83	5	0.55	Yes	Yes
4	7	77	46	0.90	Yes	Yes
5	12	56	88	1.10 (smear)	Yes	Yes
6	19	70	85	0.20/2.40 (smear)	Unknown	Unknown
7	9	53	95	0.70	Yes	Yes

* *Subjects 1 and 5*, and subjects 3 and 7, respectively, are 2 pairs of sibs. In subject 6 only the CpG island associated with the larger CGG insertion (2.4 kb) was methylated. Methylation refers to percentage of methylation of the FMR1-associated CpG island. Insertion refers to length of expanded CGG repeat in the FMR1 gene.

these problems. Subject 4 is a hyperactive child and has problems with social ambivalence. Subject 5 is a hyperactive boy. Subject 7 is a shy, hyperactive boy and reacts with tantrums to many external stimuli, especially to acoustic stimuli. Subject 1 also has mild behavior problems: he has a phobia, not categorized as typical for fragile X syndrome.

Molecular Data

Four subjects have a homogenous CGG insertion length in the FMR1 gene, ranging from 600–1,100 base pairs (Fig. 1). In these subjects, percentage of methylation of the FMR1-associated CpG island ranges from 0% (subject 1) to 95% (subject 7). Subject 6 has a mosaic mutation with a small CGG repeat insertion around 200 base pairs, and a longer smear, with mean insertion length at 2,400 base pairs. In this subject only the CpG island associated with the longer insertion is methylated. The longer insertion is found in 85% of lymphocytes. Subject 2 shows a smear of CGG repeat insertion centered around 900 base pairs, with 8% methylation. Subject 3 shows an unmethylated insertion of 550 base pairs and a light streak of methylated DNA, of a corresponding insertion length. This light streak is interpreted as 5% methylation.

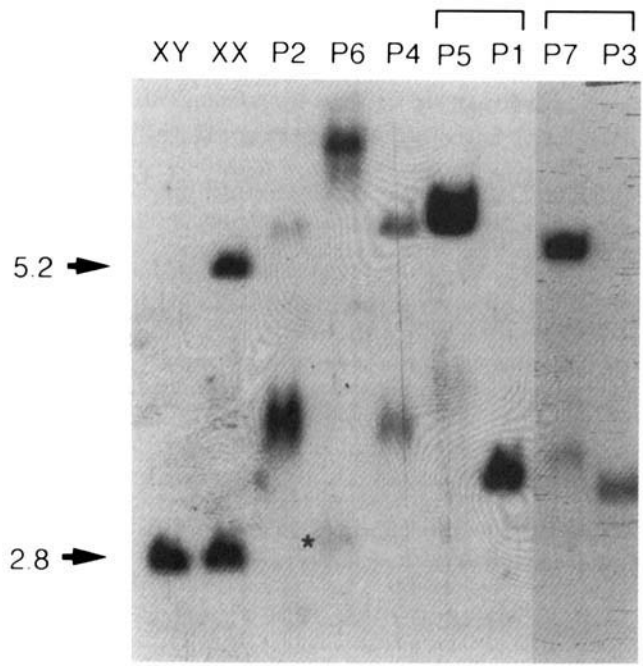

Fig. 1. Southern blot assay with probe St12.3 of DNA samples of the 7 fragile X boys. Samples were digested with *Eco*RI/*Eag*I. The light methylated band at approximately 550/600 base pairs in subject 3 (lane P3), as seen on the individual gel, cannot be seen in this reproduction; it is interpreted as 5% methylation.

Statistical Analysis

Pearson correlations between CGG insertion length at the FMR1 gene and degree of methylation of the FMR1-associated CpG island were calculated. There was no significant correlation between insertion length and degree of methylation (Pearson correlation 0.519, $P = 0.223$). In a stepwise regression analysis with IQ as dependent variable, the first step with repeat length as independent variable, could only explain a very small part of variance ($r^2 = 0.089$). When adding degree of methylation to the model, the explained variance significantly increased ($r^2 = 0.770$). This is also reflected in partial regression correlations with IQ (FSIQ) of −0.298 for repeat length and −0.860 for degree of methylation. The multivariate regression analysis is near significance ($P = 0.053$) in this small sample. Simple regression with degree of methylation as independent variable is significant ($P = 0.013$), but not significant for repeat length as independent variable ($P = 0.516$).

DISCUSSION

Since the finding of an abnormally high and unstable number of CGG repeats at the 5′ side of the FMR1 gene [Verkerk et al., 1991] as the molecular basis for fragile X syndrome, a dichotomy has been made between a so-called fragile X premutation, with an unstable CGG insertion of <600 base pairs, and a full fragile X mutation with a longer CGG insertion and concomitant methylation of the FMR1-associated CpG island. The basis for the dichotomy between premutation and mutation is the fact that an insertion of <600 base pairs is not associated with fragile X phenotype, while a longer insertion is associated with fragile X phenotype in most

males, and with a milder phenotype in approximately 1 in 2 females. The mechanism that operates is probably that an insertion of >600 base pairs is generally associated with methylation of the CpG island at the 5′ end of the FMR1 gene. This methylation does not allow transcription of the FMR1 gene, and consequently there is no production of FMR1 protein in these cells. Sutherland et al. [1991] found that this process of methylation probably occurs in early embryonic life. However, for some unknown reason, in some subjects with a mild CGG repeat insertion, methylation does not occur in all cells. This leads to a phenomenon of partial methylation, in which a fraction of the cells in a particular tissue is methylated at this site and a fraction is not. Previous studies showed that fragile X males in whom this occurs are less severely affected than males with methylation at this site in all cells. Hagerman et al. [1994] demonstrated that this incomplete methylation correlates with some production of FMR1 protein in these males. Thus, absence of methylation in a fraction of cells is one of the mechanisms explaining relatively high functioning in some fragile X males.

In the present study, incomplete methylation occurred mainly in subjects with a CGG insertion length between 550/1,100 base pairs in all lymphocytes. One subject (subject 6) showed mosaicism of 200/2,400 base pairs. We have not yet found subjects with a long insertion in all cells and incomplete methylation. One subject (subject 3) shows mild physical and behavior characteristics of fragile X, while insertion length in peripheral lymphocytes is only 550 base pairs. In this subject, methylation of CpG is present in a small proportion of cells.

The other finding is that in this range of insertion lengths, insertion length per se has no predictive value for intelligence level or penetrance of other phenotypical characteristics. By far the most important predictor of intelligence level is the degree of methylation at the FMR1-associated CpG island. Indeed, linear regression analysis shows that intelligence level correlates negatively with degree of methylation at this site.

Considering physical phenotype, the present data suggest that with the same insertion length, the degree of methylation influences the number of physical signs. Males with a lower degree of methylation are less severely affected. The data also suggest that in these mildly affected subjects, macroorchidism is the most frequent physical characteristic of fragile X syndrome. In this small sample, we did not analyze this finding statistically.

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